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ANALYSES AND DIFFERENTIATION OF BLACKLEG ISOLATES FROM THE CANADIAN PRAIRIES

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ANALYSES AND DIFFERENTIATION OF BLACKLEG ISOLATES FROM THE CANADIAN PRAIRIES

Final Report

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LIST OF ABBREVIATIONS

δ	chemical shift (ppm)
$[\alpha]_D$	optical rotation
^{13}C NMR	carbon-13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
br	broad
calcd.	calculated
COSY	COrrelated SpectroscopY
d	doublet
EI	Electron Impact
FAB	fast atom bombardment
FCC	flash column chromatography
GC	gas chromatography
h	hour
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
Hz	hertz
J	coupling constant
m	multiplet
m/z	mass / charge ratio
MHz	megahertz
min	minute
MS	mass spectrometry
MS-CI	Mass Spectroscopy-Chemical Ionization
NMR	Nuclear Magnetic Resonance
r_t	retention time
s	singlet
t	triplet
TLC	thin layer chromatography
UV	ultraviolet

a) ABSTRACT/SUMMARY

This project aims at analyzing and characterizing blackleg isolates from the Canadian Prairies to establish their variation in pathogenicity on canola and brown mustard crops.

Isolates of the blackleg fungus collected in Saskatchewan, Alberta, and Manitoba were cultured under standard conditions and their metabolite profiles were analyzed by high performance liquid chromatography. Although most of the isolates fit in the "virulent', or aggressive (PG2) group common in Canada, two isolates were found that fit in the "nonaggressive" group very common in Europe, particularly in Poland (these isolates appear to be more virulent on brown and white mustards than on canola) and suggest that there is some variation within the blackleg population present in the Canadian Prairies. We suggest that a more updated group of blackleg isolates be used for the screening of canola varieties being developed in Saskatchewan, Manitoba and Alberta institutions; new canola and mustard lines screened with updated blackleg isolates are expected to be have a broader blackleg resistant than the current varieties.

B) EXECUTIVE SUMMARY

Although in the last few years a significant number of canola varieties considered "blackleg resistant" have been registered, it is important to note that **their blackleg resistance can breakdown under propitious conditions**. That is, the blackleg pathogen, like other fungi, can quickly adapt and mutate to new forms that may overcome the resistance of newly released canola varieties. In fact, we have shown that **there are new blackleg "strains" isolated in Saskatchewan that are virulent to the traditionally resistant brown mustard**. This "new" group of blackleg fungus represents a new threat to brassica crops and poses new concerns regarding the development of canola-quality lines of brown mustard. Consequently, blackleg disease surveys with concurrent blackleg isolate characterization are extremely important and need to be conducted regularly.

Effective characterization of blackleg isolates requires simple and reliable analytical methodology. Currently, the chemical analysis of phytotoxins and related metabolites is perhaps one of the most discriminating and the least ambiguous method for differentiation of Phoma blackleg isolates, because we have established a set of **"blackleg' chemical markers** that will allow clear isolate discrimination. In this context it is necessary to clarify that chromatographic analysis utilizing only one chemical marker is not reliable and can not be used for isolate characterization or differentiation.

We have cultured in chemically defined minimal medium and analyzed by HPLC reversed phase the metabolite profiles of 110 single spore isolates obtained in the 1998-2000 Canadian Prairie surveys carried out by plant pathologists from the AAFC, Melfort Station. Results of these analyses suggested the following conclusions:

1 - The **major blackleg group contains 107 isolates** fitting in the metabolite profiles of the **"virulent" Canadian isolates group**; all these isolates produced sirodesmin PL, deacetylsirodesmin PL, phomamide, and phomalairdenone A, with sirodesmin PL being detected up to 96 h of incubation and becoming the major detectable constituent after four days. Of these 107 isolates, 63 also produced phomalide, which was detected either after 48 or 72 h of incubation. Several of these isolates produced a few new metabolites which need to be isolated and characterized. After chemical characterization of new metabolite(s), these virulent isolates might be grouped into two similar but distinct groups, one of which forms the "typically Canadian virulent group" (PG2) and another group with profiles closer to the Australian virulent isolates (PG4).

2 - The **second blackleg group is formed by two isolates** from Alberta fitting in the metabolite profiles of **"avirulent" or "nonaggressive" European isolates**; neither of these two isolates produced either phomalide, phomamide, sirodesmins, or related metabolites, but produced polanrazines and phomapyrones and showed similar chromatographic profiles to each other. These profiles clearly indicated that these two isolates did not resemble the profiles of either the virulent isolates or the avirulent isolates originating from Canada, but resembled the profiles of Polish isolates typically virulent to *B. juncea*.

3 - The **third group contains one isolate** from Alberta fitting in the **metabolite profiles of "avirulent" Canadian isolates**; this isolate produced several polyketides, including phomapyrone A but no polanrazines (dioxopiperazines); this isolate, although closer to the avirulent Polish isolates than

to the Canadian virulent isolates, was distinct from other groups. Based on the metabolite profile this isolate fits particularly well within the typical Canadian avirulent isolate group.

Our classification using HPLC metabolite profiles of isolates distinguishes clearly the virulent type isolates and is consistent with the results of pathogenicity tests. Our sub-grouping within virulent isolates using new metabolites is also consistent with pathogenicity tests. Importantly, our data showed that within the major "virulent" type an apparently "new" blackleg subgroup is present in the three prairie provinces; this particular subgroup of isolates appears to be closer to the Australian virulent blackleg isolates than the traditional Canadian virulent isolates. Because Australian type isolates appear to be more virulent to canola than the virulent Canadian isolates, the presence of these isolates in the Canadian prairies may cause further blackleg outbreaks.

On the other hand, within the "avirulent" group we can distinguish two groups, Canadian and Polish type isolates, whereas pathogenicity tests did not distinguish between these two groups. Nonetheless, we suggest that it is of great importance to develop a pathogenicity test able to distinguish between these two types, since the "Polish type" blackleg is more virulent to brown mustard and may become widespread due to the increased cultivation of new canola-quality mustard cultivars.

It is clear that the results of this project raise concerns regarding the origin of new types of blackleg. Certain "avirulent" Canadian isolates may have originated in Europe, and a subgroup of "virulent" blackleg isolates may not have originated in Canada but got here through seed brought in from Australia. However, these "new types" pose a potential threat to the *B. juncea* crops cultivated in the Canadian prairies.

In conclusion, it is important to continue with blackleg disease surveys and to establish the pathogenicity ranges of blackleg isolates distributed in the Canadian prairies. Without this information, the durability of blackleg resistance becomes unpredictable and epidemics are rather difficult, if not impossible, to forecast. Based on the results obtained during this study we make the following recommendations.

⇒ We recommend that a more updated group of blackleg isolates (containing Australian and Polish type isolates) be used to screen new canola varieties being developed in Canadian institutions. It is anticipated that such canola and mustard lines will display higher blackleg resistant than the current cultivars/lines.

⇒ Considering that HPLC metabolite profiles of blackleg isolates distinguished two subgroups within the virulent type isolates and that these conclusions are consistent with the results of pathogenicity tests, we recommend that the chemical structures of new "marker" metabolites be determined to establish if they can be used to clearly distinguish between virulent Canadian and Australian subgroups.

⇒ Finally, because there are at least two groups within the "avirulent" blackleg group, the Canadian and Polish type isolates, and currently used pathogenicity tests were unable to distinguish between these groups, we recommend the development of pathogenicity tests able to distinguish between these two types.

C) TECHNICAL REPORT

1. Objectives

1) HPLC analyses and characterization of Prairies blackleg isolates utilizing metabolic markers (blackleg isolates obtained from surveys conducted by R. Kutcher/M. Keri);

2) Comparison of HPLC results with pathogenicity testing and DNA analyses (independent project carried out by M. Keri/R. Kutcher) to establish pathogen variability and distribution across the Prairies, and appropriate pathogenicity grouping;

3) Propose new groups of blackleg isolates to screen for disease resistance.

2. Project activities, achievements, papers, and presentations

Invited lectures and invited conference presentations:

Pedras, M. S. C. **2002**. New Phytotoxins from Blackleg Fungal Isolates and Implications for Taxonomic Reclassification. 13th Crucifer Genetics Workshop, University of California, Davis, CA, March.

Pedras, M. S. C. **2002**. Mechanisms of Defence and Resistance in Plants and their Fungal Pathogens: Phytotoxin and Phytoalexin Metabolism. Brock University, St. Catherines, ON, February.

Conference presentation:

Pedras, M. S. C.; Uppala, V.; Chumala, P. B.; Cekic V. **2002** "Metabolome Analysis of Blackleg Fungal Isolates and Implications for Taxonomic Reclassification". 85th CSC Conference, June, Vancouver, BC.

Pedras, M. S. C.; Uppala, V.; Cekic, V. **2001**. " HPLC analyses of cultures of blackleg isolates from the Canadian Prairies". 2001 Meeting of the Saskatchewan Regional Group of the Canadian Phytopathological Society Saskatchewan, November, Saskatoon, SK.

Manuscript in preparation:

Pedras, M. S. C.; Uppala, V.; Cekic, V. Metabolic profiles from new blackleg isolates: culture conditions and HPLC analysis. *Canadian Journal of Microbiology*.

3. Rationale

Blackleg [caused by the fungus *Phoma lingam* (Tode ex Fr.) Desm., perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.] is one of the most damaging diseases of canola and rapeseed and poses a threat to the oilseed industry in Saskatchewan and across the prairies (Gugel & Petrie, 1992).

Common practices to prevent the spread of blackleg disease in canola and rapeseed, are crop rotations, use of certified seed, removal of infected stubble, and application of fungicides. Considering the number of blackleg resistant/tolerant Argentine canola varieties commercially available (SAF 2000 seed guide) it would appear that the once feared disease is now under control. However, it is widely known that plant pathogenic fungi can mutate and become virulent on new host plants (Knogge, 1996). That is, the plant-pathogen interaction is a dynamic process which requires continuous epidemic surveys and pathogen characterization to establish pathogen variability and subsequent strategies for its control. Currently, effective characterization of blackleg isolates require simple and reliable analytical methodology to characterize and differentiate the diverse groups of blackleg isolates (Pedras & Biesenthal, 2000).

Recent data indicates that certain blackleg isolates currently classified as *L. maculans/P. lingam* species likely belong to *P. wasabiae* species (Pedras et al., 1995; Taylor et al. 1995; Reddy, et al. 1998). That is, the "blackleg species complex" is comprised of at least two different but related species (Pedras et al., 1996), traditionally called "virulent" and "weakly virulent" strains or groups (for recent review see Williams & Fitt, 1999, Howlett et al., 2001).

We have investigated the metabolite profiles of many isolates of the blackleg fungus obtained from diverse parts of the world (part of the International Blackleg Crucifer Network collection, AAFC Saskatoon), utilizing specific culture conditions, HPLC analysis, and a set of "blackleg" markers (Pedras & Biesenthal, 2000, Pedras, 2001). Following HPLC analyses, these isolates could be placed in three main groups, irrespective of country of origin, but related with their virulence: (1) isolates producing phomamide and sirodesmins, virulent on Argentine and Polish canola (*B. napus* and *B. rapa*); (2) isolates producing indolyl dioxopiperazines, virulent on both canola and brown mustard (*B. juncea*); (3) isolates producing polyketides, weakly virulent on Argentine canola but virulent on the crucifer wasabi (*Eutrema wasabi*).

Although to date only two of the isolates virulent on both canola and brown mustard were isolated in Saskatchewan (Laird and Mayfair), these isolates are similar to the Polish blackleg isolates infecting canola in Poland (personal communication M. Jedryczka, Institute of Plant Genetics, Polish Academy of Sciences). Contrary to most blackleg isolates from Canada, isolates Laird 2 and Mayfair 2 are more pathogenic on brown mustard than on canola (Taylor et al. 1995). This "new" group of blackleg isolates represents a new threat to brassica crops and poses new concerns regarding the development of canola-quality lines of brown mustard (the fungus has obviously succeeded in overcoming the host-resistance).

Furthermore, weakly virulent isolates included in the NA1 avirulent blackleg group were reclassified as the new species *Leptosphaeria biglobosa* (Shoemaker and Brun, 2001). In addition, further sub-grouping based on diverse isolate characteristics indicate the need for reliable characterization methods and eventual revision of current nomenclature.

The chemical analysis of phytotoxins and related metabolites is perhaps one of the most discriminating and the least ambiguous method for differentiation of Phoma blackleg isolates, since we have established a **set of "blackleg' chemical markers** that will allow clear isolate discrimination (Pedras & Biesenthal, 2000). In this context it is necessary to clarify that chromatographic analysis utilizing only one chemical marker (sirodesmin PL, Badawy et al., 1991) is not reliable and can not be used for isolate characterization or differentiation.

In conjunction with pathogenicity testing and DNA analysis, HPLC analyses using blackleg marker metabolites will allow a reliable characterization of blackleg isolates from Canadian Prairie fields (survey by R. Kutcher and M. Keri, AAFC, Melfort Station). In concluding this project we can recommend a more updated group of blackleg isolates that will be of great importance for screening new canola varieties being developed in several Saskatchewan institutions.

4. Materials and Methods

4.1 General

Analytical TLC: (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5×2 cm ×0.2 mm; mobile phase was dichloromethane - methanol (90:10) and compounds were visualized under UV light and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by heating.

HPLC analysis: performed with a liquid chromatograph equipped automatic injector, and diode array detector (wavelength range 190 - 600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. All solvents were HPLC grade and used as such. Two different elution methods were employed; *method A*: linear gradient from 75% H_2O - 25% CH_3CN to 100% CH_3CN, at a flow rate 1.0 mL / min over 35 min; *method B*: isocratic elution 80% H_2O - 20% CH_3CN for 10 min, followed by gradient elutions, 80% H_2O - 20% CH_3CN to 60% H_2O - 40% CH_3CN for 10 min, and 60% H_2O - 40% CH_3CN to 25% H_2O - 75% CH_3CN for 10 min, at a flow rate 1.0 mL / min.

NMR analysis: NMR spectra were recorded on Bruker Avance 500 spectrometers; for ^1H (500 MHz), δ values were referenced to CD_3OD (CD_2HOD at 3.31 ppm), CD_2Cl_2 ($CDHCl_2$ at 5.32 ppm), $CDCl_3$ ($CHCl_3$ at 7.26 ppm), and for ^{13}C (125.8 MHz) referenced to CD_3OD (49.15 ppm), CD_2Cl_2 (54.00 ppm), or $CDCl_3$ (77.23 ppm). The multiplicities of ^{13}C NMR signals were determined utilizing J modulation experiments, HMQC and HMBC data.

HRMS analysis: mass spectra were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) or fast atom bombardment (FAB)], employing a solids and/or continuous flow probes.

4.2 General procedure for culturing blackleg isolates and analyzing extracts from fungal cultures

Agar plates from single-spore blackleg isolates (one plate per isolate), are listed in Table 2. Spores from each plate were aseptically collected and subcultured on multiple V8 agar plates (Petrie 1988), as previously reported (Pedras & Biesenthal, 1998, 2000). Pycnidiospores were collected after 15 days of growth under constant cool fluorescent light at 23 ± 2 °C. The fungal cultures were grown in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of minimal media (MM) plus thiamine (Tinline et al. 1960). Spores were inoculated at a concentration of 1×10^9 spores per 100 mL of culture medium, incubations were carried out on a shaker at 130 rpm, at 20 ± 1 °C. Production of metabolites was examined by taking 15 mL samples at various times (48, 72, 96, 168 h) and extracting with diethyl ether or ethyl acetate. The extract was dried, concentrated to dryness, and the residue was analyzed by HPLC. (12 samples per isolate).

Extracts of cultures were analyzed by HPLC utilizing two different elution methods A and B and a set of metabolic markers. This analytical method has no parallel as far as providing an efficient, simple, and reliable means for blackleg differentiation and characterization.

Upon comparison of ultraviolet spectra of peaks present on HPLC chromatograms (obtained with photodiode array detection) and those of blackleg markers, blackleg isolates from the Prairies were grouped and subsequently compared with pathogenicity grouping (communicated by M. Keri, AAFC, Melfort station) as shown on table 1.

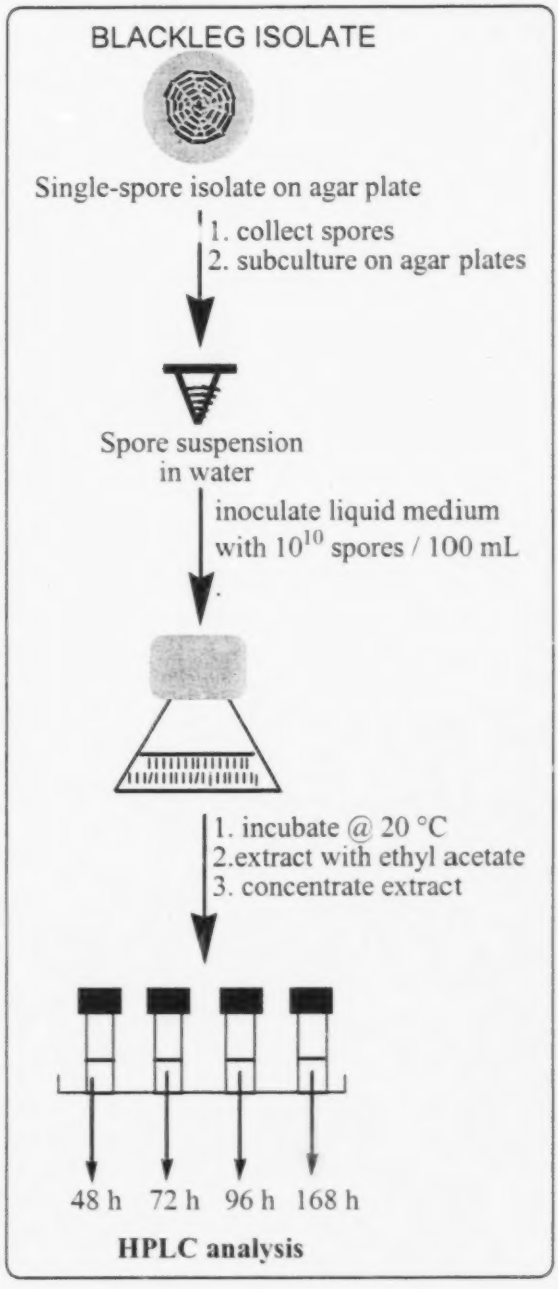

Figure 1. Flow chart for culture and analysis of blackleg

Spectroscopic analysis (namely NMR, COSY, HMQC, HMBC, and HRMS-EI, FAB, or MS-CI) was employed where necessary to confirm the chemical structures of some fungal metabolites. **Phomalide** (Pedras et al. 1993), **phomamide** and **sirodesmins** (Pedras 1998), **phomalairdenone A** (Pedras et al. 1999), **phomapyrone A** (Pedras et al. 1994), **polanrazine A** (L-valyl-L-tryptophan anhydride, i.e. **polanrazine A**, Pedras et al. 1998, Pedras and Biesenthal, 2001), and **polanrazines B-D** (Pedras and Biesenthal, 2001) were isolated and purified as reported in references cited for each compound. The purified compounds were identical in all respects, including optical rotations, to authentic samples available in our toxin library, and the purity was established to be higher than 98% by TLC and HPLC (retention times shown in table 1), and ^{1}H NMR.

5. Results and Discussion

Table 1. HPLC retention times of phytotoxins and related metabolites produced by virulent and avirulent isolates of *Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm. (general conditions described in materials and methods).

Phytotoxin or metabolite (marker compound)	Retention time ± 0.1 min A [a]	B [b]	UV_{max} (nm)
Deacetylsirodesmin PL (phytotoxin from virulent Canadian isolates)	8.0	17.8	210, 240
Phomalairdenone (phytotoxin from new [c] avirulent Canadian isolates and Polish isolates)	12.2	22.1	240
Phomalide (selective phytotoxin from virulent Canadian isolates)	30.3	32.4	210, 250
Phomamide (metabolite from virulent isolates)	7.8	17.8	230, 270
Phomapyrone A (phytotoxin from avirulent Canadian isolates)	23.2	29.6	210, 250, 340
Polanrazine A (phytotoxin from Polish isolates)	6.1	10.0	220, 280
Polanrazine B (phytotoxin from Polish isolates)	12.2	22.4	220, 280
Polanrazine C (phytotoxin from Polish isolates)	6.0	10.6	220, 280
Polanrazine D (phytotoxin from Polish isolates)	6.1	11.3	220, 280
Sirodesmin PL (phytotoxin from virulent Canadian isolates)	14.2	24.4	210, 240

[a] HPLC solvent system A, as in materials and methods.

[b] HPLC solvent system B, as in materials and methods.

[c] See Pedras et al., 1999.

Spores of 130 blackleg isolates were obtained from the AAFC, Melfort Station. Out of the 130 isolates listed in Table 2, spores of twenty isolates did not germinate and were considered to be unviable after three culture attempts. The remaining 110 isolates were grown in liquid shake cultures up to 7 days,

under standardized conditions (Pedras and Biesenthal 1998, 2000); each isolate was subcultured (chemically defined minimal medium) twice in triplicate in at least two independent experiments; the HPLC analyses showed that all metabolites were produced consistently, although quantitative variations were observed. The HPLC retention times and UV_{max} of each marker compound are reported in Table 1.

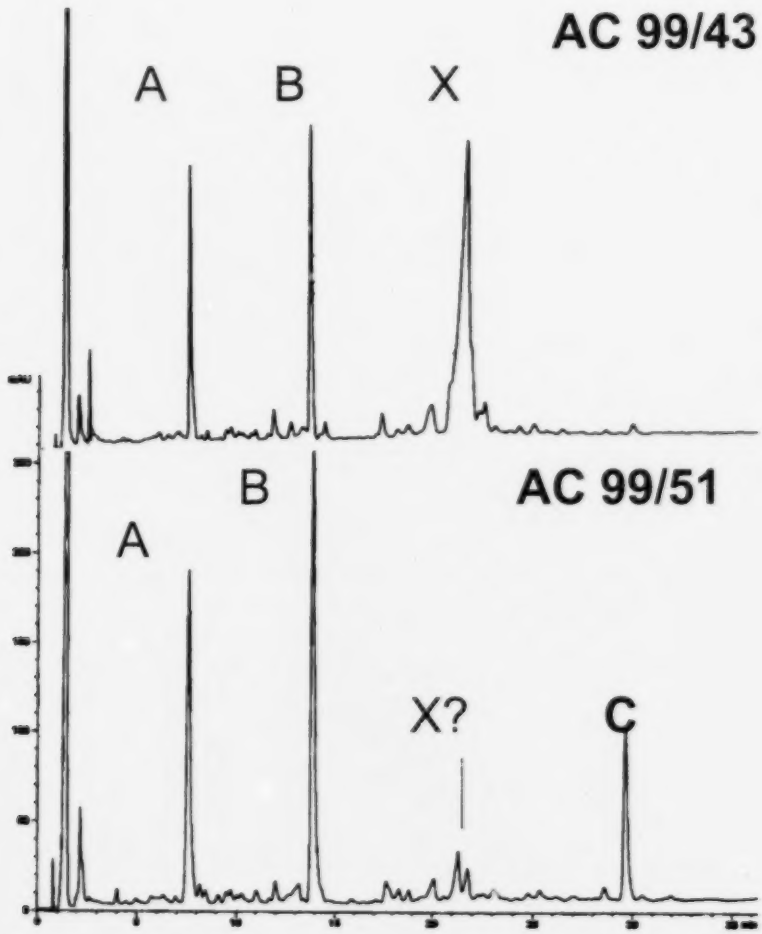

Figure 2. Typical HPLC chromatograms of virulent isolates AC 99/51 and AC 99/43 after 96 h of growth; solvent and column conditions as described in the experimental for method A; peak A identified as mixture of deacetylsirodesmin PL and phomamide; peak B identified as sirodesmin PL; peak C identified as phomalide; r_t = 21 min = peak X, chemical structure to be determined.

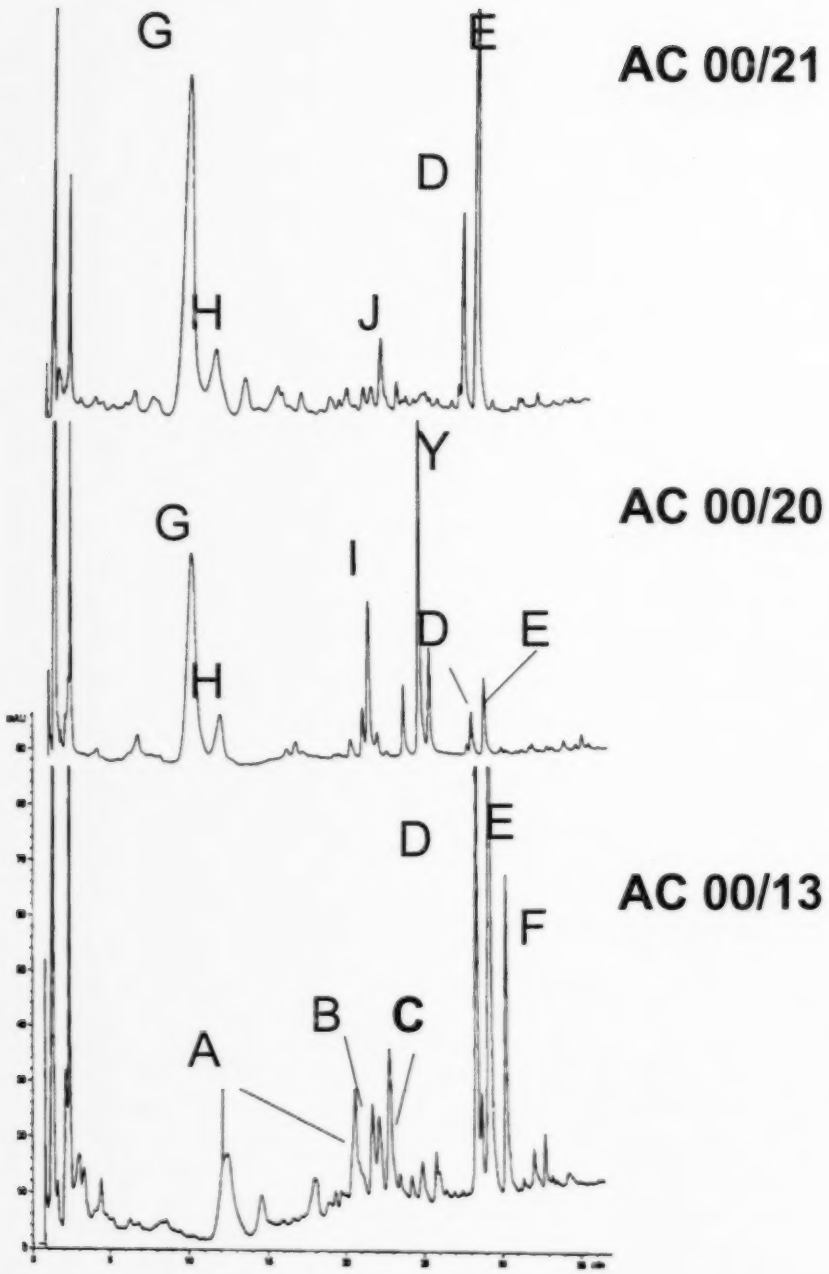

Figure 3. Typical HPLC chromatograms of avirulent isolates AC 00/13, AC 00/20, and AC 00/21 after 96 h of growth; solvent and column conditions as described in the experimental for method B; peaks A and C identified as polyketides; peak B identified as phomalairdenone; peak D identified as phomapyrone A2; peak E identified as phomapyrone A; peak F identified as phomapyrone F; peak G identified as polanrazine C; peak H identified as polanrazine D; peak I identified as polanrazine B; peak J identified as phomapyrone I; peak Y to be determined.

Figures 2 and 3 show representative chromatograms of five isolates. Based on these chromatograms and on the UV spectrum of each peak, as well as the NMR spectra of some metabolite fractions, three very different metabolite profiles were identified, as follows:

1 - The major blackleg group contains 107 isolates fitting in the metabolite profiles of the "virulent" Canadian isolates group; all these isolates produced sirodesmin PL, deacetylsirodesmin PL, and phomamide, with sirodesmin PL being detected up to 96 h of incubation and becoming the major detectable constituent after four days (Figure 2, AC 99/51). Of these 107 isolates, 63 also produced phomalide, which was detected either after 48 or 72 h of incubation (Figure 2). Several of these isolates (for example AC 99/43) produced a few new metabolites which need to be isolated and characterized (Figure 2, peak X). After chemical characterization of metabolite(s) responsible for peak X, these virulent isolates might be grouped into two similar but distinct groups, one of which forms the "typically Canadian virulent group" (PG2) and another group with profiles closer to the Australian virulent isolates (PG4).

2 - The second blackleg group is formed by two isolates from Alberta fitting in the metabolite profiles of "avirulent" or "nonaggressive" European isolates (Figure 3); neither of these two isolates produced either phomalide, phomamide, sirodesmins, or related metabolites, but produced polanrazines and phomapyrones and showed similar chromatographic profiles to each other. These profiles clearly indicated that these two isolates did not resemble the profiles of either the virulent isolates or the avirulent isolates originating from Canada, but resembled the profiles of Polish isolates typically virulent to *B. juncea*.

3 - The third group contains one isolate from Alberta fitting in the metabolite profiles of "avirulent" Canadian isolates (Figure 3); this isolate produced several polyketides, including phomapyrone A but no polanrazines (dioxopiperazines); this isolate, although closer to the avirulent Polish isolates than to the Canadian virulent isolates, was distinct from other groups. Based on the metabolite profile this isolate fits particularly well within the typical Canadian avirulent isolate group.

Our classification using HPLC metabolite profiles of isolates distinguishes clearly the virulent type isolates and is consistent with the results of pathogenicity tests carried out by Dr. M. Keri (personal communication). Our sub-grouping within virulent isolates using compound X is also consistent with pathogenicity tests.

On the other hand, within the "avirulent" group we can distinguish two groups, Canadian and Polish type isolates, whereas pathogenicity tests carried out by Dr. M. Keri (personal communication) were unable to distinguish between these two groups. Nonetheless, we believe that it would of great importance to develop a pathogenicity test able to distinguish between these two types, since the "Polish type" blackleg may become widespread due to the increased cultivation of new canola-quality mustard cultivars.

Table 2. Blackleg isolates[a] cultured and analyzed by HPLC using both solvent systems A and B: entries 1-107 represent virulent isolates with no peak at r_t = 21 min, similar to AC 99/51 on Figure 2 or with a peak X at r_t = 21 min, similar to AC 99/43 on Figure 2; entry 108 represents avirulent isolate on Figure 3; entries 109 and 110 represent avirulent isolates on Figure 3.

Entry	Isolate (origin) [b]	Phomamide, deacetylsirodesmin PL & sirodesmin PL	Phoma-lairdenone A	Phomalide	X r_t = 21 min metabolite
1.	AC 99/59 (M)	+++ [c]	+	ND [d]	+
2.	AC 99/53 (M)	+++	+	+	+
3.	AC 99/44 (M)	+++	+	+	+
4.	AC 99/49 (M)	+++	+	ND	+
5.	AC 99/40 (M)	+++	+	ND	+
6.	AC 99/43 (M)	+++	+	ND	+
7.	AC 99/76 (S)	+++	+	ND	ND
8.	AC 99/69 (M)	+++	+	ND	+
9.	AC 99/7 (M)	+++	+	+	ND
10.	AC 99/48 (M)	+++	+	+	+
11.	AC 99/36 (S)	+++	+	ND	+
12.	AC 99/38 (S)	+++	+	ND	+
13.	AC 99/16 (S)	+++	+	+	ND
14.	AC 99/25 (S)	+++	+	ND	ND
15.	AC 99/20 (S)	+++	+	ND	ND
16.	AC 99/35 (S)	+++	+	+	ND
17.	AC 99/54(M)	+++	+	+	ND
18.	AC 99/58 (M)	+++	+	+	ND
19.	AC 99/63 (M)	+++	+	ND	+
20.	AC 99/17 (S)	+++	+	+	+
21.	AC 99/13 (S)	+++	+	+	+
22.	AC 99/51 (M)	+++	+	+	+
23.	AC 99/56 (M)	+++	+	+	ND
24.	AC 00/006 (A)	+++	+	ND	ND
25.	AC 00/009 (A)	+++	+	ND	ND

26.	AC 00/012 (A)	+++	+	ND	ND
27.	AC 00/017 (A)	+++	+	+	ND
28.	AC 00/11 (A)	+++	+	+	ND
29.	AC 00/10 (A)	+++	+	ND	ND
30.	AC 00/19 (A)	+++	+	+	ND
31.	AC 99/101 (A)	+++	+	ND	ND
32.	AC 99/103 (A)	+++	+	+	ND
33.	AC 99/104 (A)	+++	+	ND	ND
34.	AC 99/105 (A)	+++	+	+	ND
35.	AC 99/110 (A)	+++	+	ND	ND
36.	AC 99/107 (A)	+++	+	ND	ND
37.	AC 99/111 (A)	+++	+	ND	ND
38.	AC 99/120 (A)	+++	+	+	ND
39.	AC 00/004 (A)	+++	+	ND	ND
40.	AC 00/005 (A)	+++	+	+	ND
41.	AC 00/S01 (S)	+++	+	ND	ND
42.	AC 00/S03 (S)	+++	+	ND	ND
43.	AC 00/S04 (S)	+++	+	+	ND
44.	AC 00/S06 (S)	+++	+	+	ND
45.	AC 00/S16 (S)	+++	+	ND	ND
46.	AC 00/S18 (S)	+++	+	ND	ND
47.	AC 00/15 (A)	+++	+	+	ND
48.	AC 00/S19 (S)	+++	+	+	ND
49.	AC 00/S24 (S)	+++	+	+	ND
50.	AC 00/S27 (S)	+++	+	+	ND
51.	AC 00/S21 (S)	+++	+	+	ND
52.	AC 00/S22 (S)	+++	+	ND	ND
53.	AC 00/S28 (S)	+++	+	+	ND
54.	AC 00/S30 (S)	+++	+	+	ND
55.	AC 00/S31 (S)	+++	+	+	ND
56.	AC 00/S32(S)	+++	+	+	ND

57.	AC 00/S39 (S)	+++	+	+	ND
58.	AC 00/S95 (S)	+++	+	+	ND
59.	AC 98/01 (S)	+++	+	+	ND
60.	AC 98/02 (S)	+++	+	+	ND
61.	AC 00/M16 (M)	+++	+	+	ND
62.	AC 00/M19 (M)	+++	+	+	ND
63.	AC 00/M07 (M)	+++	+	+	ND
64.	AC 00/S062 (S)	+++	+	+	ND
65.	AC 00/S063 (S)	+++	+	ND	ND
66.	AC 00/S072 (S)	+++	+	ND	ND
67.	AC 00/S074(S)	+++	+	+	ND
68.	AC 00/S084(S)	+++	+	ND	ND
69.	AC 00/S091 (S)	+++	+	+	ND
70.	AC 98/03 (S)	+++	+	ND	ND
71.	AC 98/04 (S)	+++	+	ND	ND
72.	AC 98/05 (S)	+++	+	ND	ND
73.	AC 98/06 (S)	+++	+	+	ND
74.	AC 98/07 (S)	+++	+	+	ND
75.	AC 98/09 (S)	+++	+	+	ND
76.	AC 98/10 (S)	+++	+	+	ND
77.	AC 98/12 (A)	+++	+	+	ND
78.	AC 98/14 (A)	+++	+	ND	+
79.	AC 98/15 (S)	+++	+	+	ND
80.	AC 98/16 (S)	+++	+	ND	ND
81.	AC 98/17 (S)	+++	+	ND	ND
82.	AC 98/19 (S)	+++	+	+	ND
83.	AC 98/20 (S)	+++	+	+	ND
84.	AC 98/22 (S)	+++	+	+	ND
85.	AC 98/26 (S)	+++	+	+	ND
86.	AC 98/27 (S)	+++	+	+	ND
87.	AC 98/28 (S)	+++	+	ND	ND

88.	AC 98/18 (S)	+++	+	ND	ND
89.	AC 98/13(A)	+++	+	ND	ND
90.	AC 98/38 (S)	+++	+	ND	ND
91.	AC 98/11 (A)	+++	+	ND	ND
92.	AC 00/M18-1 (M)	+++	+	+	ND
93.	AC 00/M19 (M)	+++	+	+	ND
94.	AC 00/M20-1 (M)	+++	+	+	ND
95.	AC 00/M37 (M)	+++	+	+	ND
96.	AC 00/M23-1 (M)	+++	+	+	ND
97.	AC 00/M33-1(M)	+++	+	+	ND
98.	AC 00/M34 (M)	+++	+	ND	ND
99.	AC 00/M36-1(M)	+++	+	+	ND
100.	AC 00/M53 (M)	+++	+	ND	ND
101.	AC 00/M47-1 (M)	+++	+	+	ND
102.	AC 00/M51 (M)	+++	+	+	ND
103.	AC 00/M42-1 (M)	+++	+	+	ND
104.	AC 00/M54 (M)	+++	+	+	ND
105.	AC 00/M39 (M)	+++	+	ND	ND
106.	AC 00/M26(M)	+++	+	+	ND
107.	AC 00/M52-1(M)	+++	+	ND	+

Entry	**Isolate**	**polanrazines A-G**	**phomapyrones A-C**	**phomalairdenones A-C**
108.	AC 00/013 (A)	ND	+++	+++
109.	AC 00/020 (A)	+++	+++	+++
110.	AC 00/021 (A)	+++	+++	+++

[a] Isolates collected by Drs. M. Keri and R. Kutcher, AAFC, Melfort Station.

[b] A = Alberta; M = Manitoba; S = Saskatchewan. [c] HPLC analytical conditions as reported in materials and methods; +++ = all metabolites present. [d] + = metabolite present.

[e] ND = metabolite not detected.

Table 3. Number of new blackleg isolates analyzed according to province of origin and classification

Province	**Number of isolates analyzed**;[a] group according to metabolite profiles
Alberta	**25**; 22 virulent (PG2 and PG4), 3 avirulent
Manitoba	**33**; all virulent (PG2 and PG4)
Saskatchewan	**52**; all virulent (PG2 and PG4)

[a] Total number of isolates grown 130 but from these only 110 could be subcultured.

6. Conclusions

The culture conditions and HPLC analysis utilizing the chemical markers shown in table 1, allowed the differentiation of "Phoma blackleg" isolates into three groups. The three different groups are comprised of: (i) Canadian virulent isolates (PG2), producing as characteristic metabolites phomamide, deacetylsirodesmin PL, sirodesmin PL and phomalairdenone A; (ii) avirulent isolates, producing a variety of polyketides and no indolyl dioxopiperazines; (iii) "avirulent" Polish isolates (containing also Canadian isolates Laird 2 and Mayfair 2) producing as characteristic metabolites indolyl dioxopiperazines, such as polanrazines and polyketides as phomapyrone A (NAx).

Most importantly, our HPLC analyses have shown production of several new metabolites by "virulent" isolates indicating a variation among these isolates, irrespective of isolate origin. Our chemical analysis data suggest that these isolates can be differentiated into two main subgroups, the Canadian/European group (PG2) and another group similar to Australian isolates (PG4). Importantly, these results are consistent with pathogenicity tests; however, results of DNA analyses of these isolates (apparently a much smaller number was analyzed) will not be made available to us before that work is published (M. Keri, personal communication in Dec. 2002). We expect to be able to compare our HPLC results and conclusions at that time, however we do not expect that DNA analyses will be as consistent and provide as specific conclusions as our metabolome analysis.

It is clear that the results of this project raise further concerns regarding the origin of certain "avirulent" Canadian isolates (AC 00/020 and AC 00/021) and their potential threat to the *B. juncea* crops in Canada, since our previous work showed that isolates with related metabolite profiles were more virulent on *B. juncea* than on *B. napus* (Pedras, 2001). In addition, the subgroup of "virulent" isolates closer to the Australians isolates (e.g. AC 99/43) than to the traditional "virulent" Canadian isolates may not have originated in Canada but got here through seed brought in from either Australia or European countries.

In conclusion, it is important to continue with blackleg disease surveys and to establish the pathogenicity ranges of blackleg isolates present in the Canadian prairie crops. Without this information, the durability of blackleg resistance becomes unpredictable and epidemics are rather difficult, if not impossible, to forecast. We recommend that a more updated group of blackleg isolates (e.g. AC 00/020 and AC 00/021, and AC 99/43) be used to screen new canola varieties being developed

in several Saskatchewan institutions. It is anticipated that new canola and mustard lines screened with updated blackleg isolates will display higher blackleg resistant than the current varieties.

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8. Suggestions for Further Development

1. - Our classification using HPLC metabolite profiles of blackleg isolates distinguishes clearly the virulent type isolates and is consistent with the results of pathogenicity tests; our sub-grouping within "virulent" Canadian and Australian type isolates using compound(s) X is also consistent with pathogenicity tests.

   ⇒ Consequently, we recommend that compound(s) X be isolated and its chemical structure determined to establish if X can be used as a chemical marker to distinguish the two subgroups

2. - We can distinguish two groups within the "avirulent" group, the Canadian and Polish type isolates, however currently used pathogenicity tests were unable to distinguish between these two groups, but the "Polish type" blackleg may become widespread and cause great yield losses due to the increased cultivation of new canola-quality mustard cultivars.

   ⇒ Consequently, we recommend the development of pathogenicity tests able to distinguish between these two types.

9. Information of Benefit to Producers, Processors, or Government

It is clear that the results of this project raise further concerns regarding the threat of certain "avirulent" Canadian isolates (AC 00/020 and AC 00/021) to the *B. juncea* crops in Canada.

In addition, the subgroup of "virulent" isolates closer to the Australians isolates (e.g. AC 99/43) than to the traditional "virulent" Canadian isolates suggests that virulent type isolates may be emerging and can lead to higher yield losses of lines/cultivars currently known as blackleg resistant.

⇒ Consequently, we recommend that a more updated group of blackleg isolates containing new isolates AC 00/020 and AC 00/021, and AC 99/43, be used to screen new canola varieties being developed in several Saskatchewan institutions. It is anticipated that new canola and mustard lines screened with updated blackleg isolates will display higher blackleg resistant than the current varieties.

D) PERSONNEL

Dr. Venkatesham Uppala, full-time postdoctoral fellow (100% of research work committed to this project) was hired in December and took up position on Feb 19. 2001

Ms. Vera Cecik, a part-time technical assistant, hired on April 2002 terminated November 2002

E) EQUIPMENT

No equipment was purchased, expenses covered disposable supplies (including HPLC columns and disposables), chemicals and solvents, and minor glassware.

F) PROJECT DEVELOPED MATERIALS

Optimized protocols for extraction and HPLC analyses as described in this report.

G) PROJECT PHOTOS

No photos were obtained for this project.

H) ACKNOWLEDGEMENTS

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The scientific work was carried out by Dr. Uppala, under supervision and direction of Professor Pedras and with the technical assistance of Ms. Vera Cekic.

We thank K. Brown and K. Thoms at the Department of Chemistry, University of Saskatchewan, for technical assistance obtaining NMR and MS data, respectively.

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